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Hepatitis C Virus Genotypes Implicated in Mixed Cryoglobulinemia

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Recent reports suggest that hepatitis C virus (HCV) might be a causative agent of mixed cryoglobulinemia. To determine whether the HCV genotype is a factor implicated in the onset of cryoglobulinemia, genotyping by direct sequencing of polymerase chain reaction products of the 5′ non coding region was carried out among 45 HCV-infected patients. Genotypes 1 and 2 were found more prevalent in symptomatic cryoglobulinemia patients. Due to the presence of genotypes 4 and 5 found in this panel of French patients (9.3%), HCV genotyping based on sequence determination is recommended. ***J. Med. Virol. 54:20–25, 1998.***

KEY WORDS: 5′ non coding region; genotyping; UPGMA

INTRODUCTION

Hepatitis C virus (HCV) [Choo et al., 1989] is implicated in most cases of nonA-nonB hepatitis. HCV infection is a silent and insidious disease that leads to chronic hepatitis with long-term complications: cirrhosis and hepatocellular carcinoma [Di Bisceglie et al., 1991; Yano et al., 1993].

At least six major genotypes and numerous subtypes of HCV have been identified by sequence comparison of either the full-length genome or parts of it (core, NS3, NS5 or E1 regions) [Simmonds et al., 1994a; Smith et al., 1997]. Analysis of the 5′ non coding region (5′NCR) also enables HCV genotyping and can discriminate among the six major types and a few subtypes due to the marked nucleotide conservation in this subgenomic region [Stuyver et al., 1993, 1994; Simmonds et al., 1994b; Davidson et al., 1995].

The proposed consensus taxonomic classification of HCV was based on genotype determination [Simmonds et al., 1994a], but little is known about the clinical relevance of such a classification. Several reports have suggested that a specific HCV genotype could influence pathogenesis: genotype 1 or 1b also seems to be more resistant to interferon treatment [Yoshioka et al., 1992; Nousbaum et al., 1995]; genotype 1b seems to be more frequently implicated in cirrhosis [Nousbaum et al., 1995]; genotype 2 has been associated with a higher hepatic inflammatory score [Lau et al., 1995]; genotypes 1 and 2 have been associated with higher alanine aminotransferase (ALT) levels than genotype 3 [Lau et al., 1995].

Recent data suggest strongly that HCV might be a causative agent of mixed cryoglobulinemia (MC) [Ferri et al., 1991; Misiani et al., 1992]. The main biological marker is an abnormal immunoglobulin (Ig) present in the sera of patients and which precipitates in the cold. Cryoglobulins are classified according to their Ig composition [Brouet et al., 1974]; type I cryoglobulins are monoclonal, type II contains a mixture of polyclonal Ig with a monoclonal component, and type III are polyclonal; types II and III are called mixed cryoglobulins.

Since the onset of MC can shift chronic HCV infection from a silent to a severe clinical presentation, determination of the HCV genotype(s) implicated in MC is of great clinical interest: 1) if MC is found to be associated with specific HCV genotypes, it would permit physicians to predict the risk of cryoglobulinemia during chronic HCV infection; 2) because chronic HCV infection associated with MC seems to be less sensitive to interferon treatment [Misiani et al., 1994], HCV

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genotype(s) implicated in MC might be poor-interferon-responder types [Kanai et al., 1992; Yoshioka et al., 1992; Chemello et al., 1994; Nousbaum et al., 1995].

METHODS

Patient Selection

Forty-five French patients were selected in a case-control study from 1991 to 1993: 11 with clinically symptomatic mixed cryoglobulinemia (SMC) and 34 controls (9 with asymptomatic cryoglobulinemia and 25 without cryoglobulinemia). Patients were recruited based on their positive HCV-serology (ELISA III, Ortho Diagnostic Systems; RIBA III, Chiron Corp.) and HCV-RNA by reverse transcriptase-polymerase chain reaction (RT-PCR). They were negative for HIV-serology and hepatitis B surface antigen. Clinical features and serum samples for biochemical and virological tests were collected before treatment [Cohen et al., 1996]. Cryoglobulinemia detection was undertaken as described elsewhere [Cohen et al., 1996]. Briefly, blood samples were drawn using a prewarmed Vacutainer system and kept at 37°C until clotted. The tubes were then centrifuged at 2,000*g* for 10 mn at 37°C. Sera were cleared by centrifugation at 2,000*g* for 10 mn at 37°C and then stored at 4°C for 1 week to make certain that late cryoprecipitation did not go undetected. The reversibility of the cryoprecipitation was tested by rewarming an aliquot of the precipitated serum. After isolation and washing three times with salin buffer, the components of the cryoprecipitate dissolved in a salin buffer were determined by automated immunonephelometric assay (BNA Behring, Marburg, Germany) and characterized by immunofixation electrophoresis (Sebia, Issy-les-Moulineaux, France).

HCV RNA RT-PCR and Direct Sequencing of the PCR Product (5′NCR)

Viral RNA was extracted then first-strand cDNA synthesis was carried out using a reverse primer CV320: CGGTCTACGAGACCTC. Hot start PCR [Chou et al., 1992] was performed with 5 μl of the cDNA, using a set of conserved primers of the 5′NCR (biotinylated sense primer CV49b: GAGGAACTACTGTCTTCACG; antisense primer CV293: ACTCGCAAGCACCCTATCAG). PCR amplification product were sequenced on both strands after separation of the single strands using Dynabeads (Dynal), as described previously [Cohen et al., 1996].

Sequence Analysis

Multiple sequence alignment was carried out with either CLUSTAL W [Thompson et al., 1994], or the PILEUP module of the Genetics Computer Group package (GCG, Madison, Wisconsin) [Feng and Doolittle, 1987; Higgins and Sharp, 1989; Needleman and Wunsch, 1970] on sequences from −244 to −69 as in [Simmonds et al., 1993]. The 20 non-identical sequences were subjected to phylogenetic analysis (Table I). Prototype sequences of known genotypes were included in the alignment to facilitate genotype assignment, these sequences (from GenBank) are identified in the figures by their mnemonics.

TABLE I. HCV Genotype Assignment of the 5′NCR Sequences

Non-identical sequences	Isolates	Genotype assignment
1	FR11, FR13	
2	FR9	1a
3	FR13C, FR49C	
4	FR117	
5	FR2, FR4C, FR6, FR7, FR10C, FR12, FR14, FR15C, FR23_2, FR24C, FR25C, FR33C, FR34C, FR53C, FR78C, FR130, FR132	
6	FR17C	1b
7	FR24, FR25	
8	FR8	
9	FR133C	
10	FR21	
11	FR14C, FR31C	
12	FR3C, FR6_2	2
13	FR1, FR1_2, FR20	
14	FR4	3
15	FR23	
16	FR119C	
17	FR15	
18	FR26	4
19	FR115C	
20	FR22	5

The GenBank accession numbers of the sequences reported in this paper are U51746 to U51788.

Phylogenetic analyses were carried out using either the NEIGHBOR-JOINING [Saitou and Nei, 1987] module of CLUSTAL W [Thompson et al., 1994] or programs of the PHYLIP package [Felsenstein, 1993]. In the latter case, four different methods were used to reconstruct phylogenetic trees which were then compared. Nucleotide distances were estimated using DNADIST, and phylogenetic trees were then calculated using NEIGHBOR with two options 1) Neighbor-Joining [Saitou and Nei, 1987] or 2) UPGMA (unweighted pair-group method using arithmetic averages [Sneath and Sokal, 1973]). Alternatively, two character-based programs were evaluated: 3) DNAPARS (parsimony method) and 4) DNAML (maximum likelihood method [Felsenstein, 1981]). The entire procedure was repeated with 1,000 bootstrap replications of the data. Phylogenetic trees were plotted using TREE TOOL [Maidak et al., 1994] or NJ PLOT [Thompson et al., 1994]. Sequences were ranked using the Simmonds consensus classification [Simmonds et al., 1994a].

Statistical Analyses

Symptomatic mixed cryoglobulinemia vs. asymptomatic patients were compared. The Mann-Whitney U-test was used for numerical data. Sex and HCV genotype distributions were compared between groups using Fisher's exact test. For comparison of HCV

TABLE II. Distribution of HCV Genotypes

HCV genotype	SMC (%)	Controls (%)	Total (%)
1	8 (72.7)	21 (65.6)	29 (67.4)
2	3 (27.3)	1 (3.1)	4 (9.3)
3	0	6 (18.7)	6 (14)
4	0	3 (9.4)	3 (7)
5	0	1 (3.1)	1 (2.3)
n	11	32	43

SMC: symptomatic mixed cryoglobulinemia.
Controls: positive for HCV RNA with no cryoglobulinemia or with asymptomatic cryoglobulinemia.
n: number of patients.

genotypes distribution between groups, two comparisons were done: 1) each HCV genotype was individually tested, 2) genotypes 1 and 2 were grouped in one class and other genotypes (3 to 5) in the other, since preliminary studies showed that patients with MC syndromes seem to be infected with HCV genotypes 1 and 2 [Galli et al., 1995; Mazzaro et al., 1995; Silvestri et al., 1996; Sinico et al., 1995; Willems et al., 1994; Zignego et al., 1996].

RESULTS

Clinical and Biochemical Features

The SMC group was significantly older than the control group, with a mean age: 63 years vs. 47.4 years, respectively ($P < 0.005$), and a higher prevalence of females ($P < 0.04$). No statistical difference between ALT levels could be established with the Mann-Whitney nonparametric U-test, despite a trend towards higher mean ALT levels in the SMC group (data not shown).

Detection of HCV RNA by RT-PCR and Direct Sequencing of PCR Amplification Products

Of the 45 samples, two could not be sequenced because of too small amounts of PCR products. Genotype determination and statistical analyses (Table II) were thus made on 43/45 samples. Seven of 43 isolates (16.3%) had sequence ambiguities at some base positions. Because these ambiguities were reproducible and found in both strands, they were interpreted as indicating the presence of two or more different HCV genomes in the serum sample. Nevertheless, in the majority of cases, the alternative base did not change the genotype assignment. The only exceptions were the FR8 and FR117 isolates. The alternatives base at position −99 (A instead of G) might give genotype 1a instead of 1b for FR8 and vice versa for FR117. In these cases, the sequence from only the predominant HCV strain (i.e., 1b and 1a for FR8 and FR117, respectively) was retained for genotyping (Fig. 1) and statistical analysis (Table II).

Comparison of Phylogenetic Programs for HCV Genotyping

Five commonly used phylogeny programs were tested on a subset of 5′NCR sequences of known genotypes [Smith et al., 1995]. Four of these gave similar tree's topologies (NEIGHBOR-JOINING module of

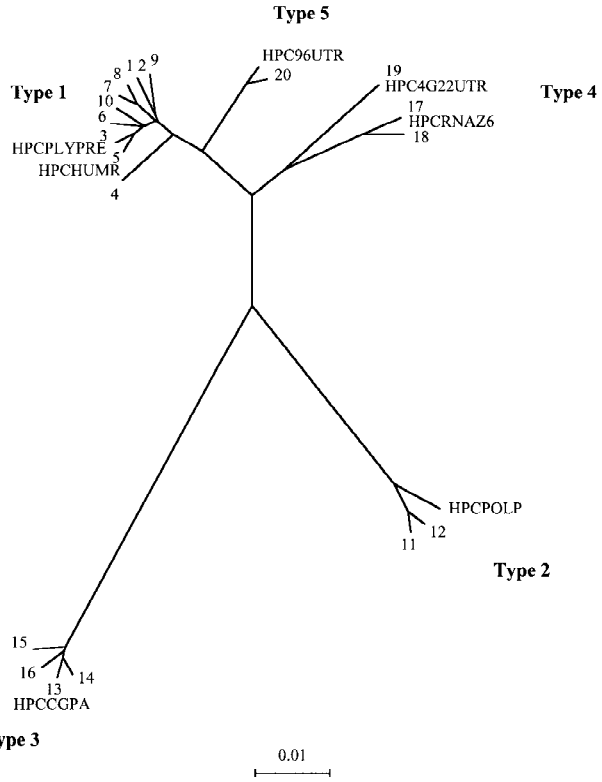

Fig. 1. Phylogenetic tree of HCV 5′NCR sequences using UPGMA. Sequences are numbered as in Table I. HCV genotype assignment is indicated in boldfaced type.

CLUSTAL W, NEIGHBOR option neighbor joining of PHYLIP, DNAPARS and DNAML). All four of these programs split genotype 4 sequences into two distinct phyla in 5′NCR (data not shown), contrary to earlier reports in the NS5 and E1 regions [Stuyver et al., 1994]. Only UPGMA (NEIGHBOR option UPGMA of PHYLIP) showed one unique phylum for genotype 4; this latter program was retained for this study (Fig. 1). Introduction of the 5′NCR of known genotypes in the phylogenetic analysis process allowed easy determination of the genotype associated with each cluster (Fig. 1). HCV genotype could thus be assigned unambiguously for all the 43 isolates.

HCV Genotype Distribution and Statistical Analyses

The HCV genotype distributions are summarized in Table II: no HCV genotype 6 was found among these French patients who live in the Paris area. Three of 43 and 1/43 were found to be genotypes 4 and 5, respectively.

The prevalence of HCV genotype 2 was significantly higher in the symptomatic mixed cryoglobulinemia group ($P < 0.05$), no statistical difference was found between groups for the other HCV genotypes when tested individually. However, genotypes 3, 4 and 5 were found only in the control group, that is genotypes 1 and 2 were found significantly more often in the

symptomatic mixed cryoglobulinemia group (100% vs. 65.6%, $P < 0.05$) when they were grouped (see Methods).

DISCUSSION

Analysis of the 5′NCR Sequences and Genotype Determination Using Different Phylogenetic Programs

The 5′NCR of the HCV genome is the most conserved region among different HCV variants worldwide [Simmonds et al., 1993, 1994b]. Indeed, it is so well conserved that it cannot be used to distinguish between the HCV subtypes, nevertheless, 5′NCR analysis can readily recognize the six major genotypes [Davidson et al., 1995; McOmish et al., 1994; Simmonds et al., 1993; Stuyver et al., 1993] based upon type-specific patterns or signatures [Smith et al., 1995; Stuyver et al., 1994].

Other easier and commercially available techniques for HCV typing were not used in this study because they are less accurate than direct sequencing of PCR products. The Line probe assay misclassify up to 17% sera [Smith et al., 1995]. The type-specific primer HCV PCR [Okamoto et al., 1992] is inadequate for genotyping samples from the United States [Lau et al., 1995]. The HCV type cannot be determined by NS4 serology in 15% [Pawlotsky et al., 1995] and 29.4% of HCV patients in France [Leruez-Ville et al., in press]. The accuracy of restriction fragment length polymorphism (RFLP) typing on 5′NCR was estimated to be 97% in an international survey [Smith et al., 1995], but the actual rate for French samples could be estimated at only 90.7% (39/43) from our study (data not shown).

We obtained sequence data for 43/45 PCR-positive samples (95.6%). Mixed infection should give rise to ambiguities at certain base positions. Indeed, we observed such ambiguities in 7/43 isolates (16.3%). But only two isolates seemed to be true mixed infections (4.7%), which is concordant with what was reported by Nousbaum et al. (5.4%) [Nousbaum et al., 1995]. The other isolates should be considered as quasispecies variants. Quasispecies variants were described previously on HCV coding sequences [Martell et al., 1992; Enomoto et al., 1994; Koizumi et al., 1995], we demonstrated here that such quasispecies sequence variations also exist in 5′NCR in 11.6% of the isolates.

During this study, several widely used phylogenetic programs were tested. The aim was not to compare them for the reconstruction of HCV evolutionary history, which remains a difficult and controversial topic [Hillis et al., 1993, 1994; Kuhner and Felsenstein, 1994; Smith et al., 1997; Edwards, 1995], but only to find out if one of them might be recommended as a tool for routine HCV genotyping, i.e., to recognize sequence type-specific patterns described in HCV 5′NCR [Simmonds et al., 1993, 1994b; Stuyver et al., 1993, 1994; McOmish et al., 1994; Davidson et al., 1995]. Most of the algorithms (maximum parsimony, maximum likelihood, neighbor-joining) failed to properly rank type 4 5′NCR sequences in one unique phylum. In essence, HCV 5′NCR sequences violate one of the major assumptions [Felsenstein, 1993] of these algorithms: independence of sites evolution. Parallel mutations have indeed probably occurred since HCV 5′NCR is constrained in a secondary structure [Brown et al., 1992; Simmonds et al., 1993; Smith et al., 1995]. Thus, we favor the use of UPGMA (i.e. NEIGHBOR with option UPGMA from the PHYLIP package) for HCV genotyping on 5′NCR sequences because it seems to be more robust to violations of the independence of sites evolution.

Genotype Distribution

Genotypes 1, 2 and 3 were the most prevalent (90.7%), in concordance with other studies from European regions [Stuyver et al., 1994; Davidson et al., 1995; Galli et al., 1995; Nousbaum et al., 1995; Pawlotsky et al., 1995], but other genotypes cannot be ignored. We found 4/43 (9.3%) to be genotypes 4 or 5 which is concordant with Stuyver et al., [1994] who reported genotype 4 and 5 prevalences 5–15% in Benelux, but these values (Table II) are clearly different from the 0.5% reported for Northern Europe [Davidson et al., 1995]. This discrepancy in HCV genotype distribution between neighboring geographical regions, i.e., a higher prevalence of "African genotypes" [Stuyver et al., 1994], could be due to the closer historical relationship that France and the Benelux countries share with African nations and the immigration flow rate. Due to the relatively high prevalence of genotypes 4 and 5 in France, rapid HCV typing methods should be evaluated carefully [Leruez-Ville et al., in press].

Association of Chronic HCV Infection and Mixed Cryoglobulinemia

Our data showed that SMC is associated with HCV genotype 2 (27.3% vs. 3.1%, Table II, $P < 0.05$) as it has been demonstrated for Italian patients [Zignego et al., 1996], and that SMC patients seemed to be infected only with HCV genotypes 1 and 2 (100% vs. 68.7%, Table II, $P < 0.05$, Fisher's exact test). But as these data were established with a rather small group of 11 SMC patients, these statistical findings have to be confirmed on larger series or meta-analysis studies since SMC is rare. Nevertheless these results are in accord with other studies [Willems et al., 1994; Galli et al., 1995; Mazzaro et al., 1995; Sinico et al., 1995; Silvestri et al., 1996; Zignego et al., 1996], showing that HCV 1 and 2 seemed to be the only genotypes associated with SMC. The hypothesis that HCV genotypes implicated in SMC might be poor-interferon-responder genotypes is supported by our data, since genotype 1b infections are considered to be more resistant to interferon [Yoshioka et al., 1992; Nousbaum et al., 1995] and are predominant in the SMC group (63.6% vs. 47%, NS).

Genotyping seems indeed useful to predict the development of SMC during chronic HCV infection contrary to the conclusions of two recent studies [Willems et al., 1994; Pawlotsky et al., 1995]. While a simple difference in geographical recruitment could account for the difference in HCV genotypes distribution comparing to

the study of Willems et al. [1994], such an argument cannot be evoked for the discrepancy with Pawlotsky's study [Pawlotsky et al., 1995] which concern a French sample population like ours. In the latter study, however, the investigators do not differentiate between symptomatic and asymptomatic cryoglobulinemia patients, this omission could have hindered a statistical difference. Multivariate analyses will however be necessary to assess definitively the role of HCV genotype, and to determine the part of the different factors beside HCV genotype (age, sex, ethnic origin) highlighted in our study. Future studies, however, will have to differentiate among the three subgroups: symptomatic, asymptomatic cryoglobulinemia and chronic HCV-infected patients without cryoglobulinemia, to address correctly these questions.

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